Systematic Botany (2004), 29(3): pp. 511–517

Pucara (Amaryllidaceae) Reduced to Synonymy with *Stenomesson* on the Basis of Nuclear and Plastid DNA Spacer Sequences, and a New Related Species of *Stenomesson*

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Communicating Editor: James F. Smith

ABSTRACT. *Pucara leucantha* is transferred to *Stenomesson* as ***Stenomesson leucanthum*** based on the phylogenetic position of *Pucara* resolved by nuclear and plastid DNA sequences. An allied species, ***Stenomesson chloranthum***, is described from the Departments of Amazonas and Cajamarca in Peru, but at lower elevations. Both of these species release their pollen in tetrads, unique within Amaryllidaceae, and have tri-lobed stigmas, unique within *Stenomesson*.

Meerow et al. (1986) described the first known occurence of mature pollen in tetrads within the Amaryllidaceae for a species they determined as *Stenomesson elwesii* Macbr. from collections by Paul Hutchinson. Meerow later had the opportunity to examine the type of *S. elwesii* [now *Clinanthus elwesii* (Macbr.) Meerow] at Kew, and it became obvious that the name was misapplied by Meerow et al. (1986). The identity of the Hutchinson collections remained unknown. While it clearly bore resemblance to a species of *Stenomesson* sensu stricto (Meerow et al. 2000), the palynological apomorphy and the low elevation of the collection were unusual.

New collections from the Department of Amazonas in Peru in 1999 and 2001 were identified as representing both this unidentified species and the sole species of *Pucara* Rav., *P. leucantha* Rav. (Ravenna 1972), allowing more detailed study. We observed that *P. leucantha* has leaf morphology similar to the undescribed species, and also releases its pollen in tetrads. In this paper, we show that the phylogenetic position of these two species based on DNA sequences leaves little doubt that the genus *Pucara* should not be recognized as distinct from *Stenomesson* (sensu Meerow et al. 2000). We further describe the misidentified taxon as a new species of *Stenomesson*, allied to the erstwhile *P. leucantha*.

MATERIALS AND METHODS

DNA Sequencing and Alignment. SAMPLING. Genomic DNA was extracted from silica gel dried leaf tissue of the taxa listed in Appendix 1 as described by Meerow et al. (2000).

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING PROTOCOLS. The plastid *atpß-rbcL* spacer was amplified and sequenced using the primers and polymerase chain reaction (PCR) protocol of Chiang et al. (1998). Amplification of the ribosomal DNA ITS1/5.8S/ITS2 region was accomplished using flanking primers (18S, 26S) AB101 and AB102 (Douzery et al. 1999), and the original White et al. (1990) internal primers ITS2 and 3 to amplify the spacers along with the intervening 5.8S gene as described by Meerow et al. (2000). All PCR amplifications were performed on an ABI 9700 (Perkin-Elmer Applied Biosystems, Foster City, California, USA).

Amplified products were purified using QIAquick (Qiagen, Valencia, California, USA) columns, following manufacturer's protocols. Cycle sequencing reactions were performed directly on purified PCR products on the ABI 9700, using standard dideoxy cycle protocols for sequencing with dye terminators on either an ABI 310 or ABI 3100 automated sequencer (according to the manufacturer's protocols; Applied Biosystems, Foster City, California, USA).

SEQUENCE ALIGNMENT. Both the ITS and *atp-rbcL* spacer matrices were readily aligned manually using Sequencher 4.1 (Gene Codes, Ann Arbor, Michigan, USA). We also used Clustal X (Higgins and Sharp 1988; Thompson et al. 1997) to align the sequences as a check against our manual alignments to help highlight any ambiguous regions. The alignments and the parsimony trees used in this paper can be accessed at TreeBase (study accession number S1037, matrix accession numbers M1762–M1765).

Phylogenetic Analyses. Both DNA sequence matrices consisted of 18 taxa (Appendix 1) representing the Andean tetraploid clade resolved by Meerow et al. (2000) with ITS sequences. Four tribes are represented: Clinantheae, Eustephieae, Hymenocallideae, and Stenomesseae. The Eustephieae (*Eustephia darwinii* and *Chlidanthus fragrans*) were used as outgroup, as this tribe is sister to the rest of the clade (Meerow et al. 2000).

Aligned matrices were analyzed using the parsimony algorithm of PAUP* for Macintosh (version 4.0b10; Swofford 1998), with the MULPARS option invoked. Tree branches were retained only if unambiguous support was available (i.e., branches were collapsed if the minimum length = 0). Gaps were coded as missing characters in the initial analyses, but a gap matrix was also constructed from the indel-rich plastid spacer region using the program PAUP-GAP (Anthony Cox, formerly RBG Kew), which applies a strict interpretation of gaps (i.e., only gaps of equal length are considered homologous among taxa). As Kelchner (2000) has pointed out, indels within plastid non-coding regions are frequently as, if not more, informative than the nucleotide sequences themselves. This binary matrix was added to the sequence alignment and analyzed in combination. For all matrices, a branch and bound (Hendy and Penny 1982) search was conducted under the Fitch (equal) weights (Fitch 1971) criterion with simple addition sequence.

We also combined the two data matrices, opting for the "total evidence" approach (Dubuisson et al. 1998; Seelanan et al. 1997). However, before combining the ITS and *atpß-rbcL* spacer data sets, we performed partition homogeneity tests on the matrices (Farris et al. 1994, 1995) to assess the degree of congruence between them. One thousand heuristic searches were conducted for each test, each with 10 random addition replications, saving no more than 20 trees from each for TBR branch swapping.

Internal support was determined by bootstrapping (Felsenstein 1985; 5000 heuristic replicates with simple addition, TRB branch-swapping, saving 20 trees per replicate) and calculation of Bremer (1988) decay indices (DI) using the program TreeRot v. 2.1 (Soren-

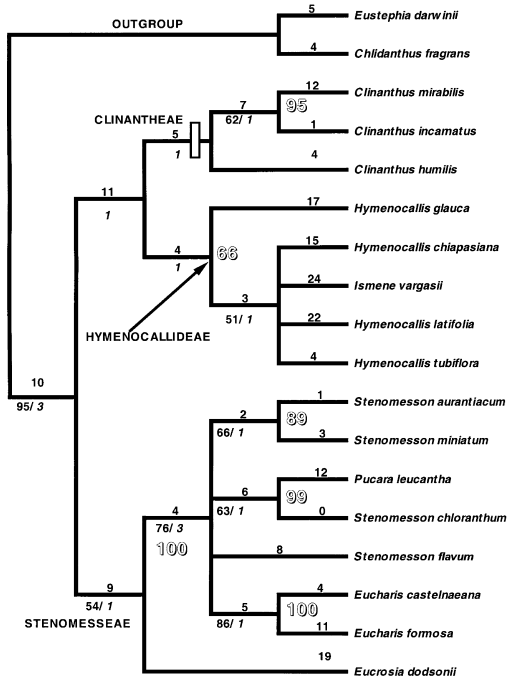

FIG. 1. One of four equally most parsimonious trees found by a cladistic analysis of the plastid *atpß-rbcL* spacer region across 18 species of Amaryllidaceae. Numbers above branches are branch lengths. Numbers below branches are bootstrap support percentages/decay indices (italic). Large shadowed numbers adjacent to nodes are ''clade credibility'' scores from 500,000 generations of Bayesian analysis. A vertical white bar indicates a branch that collapses in the strict consensus of all four trees.

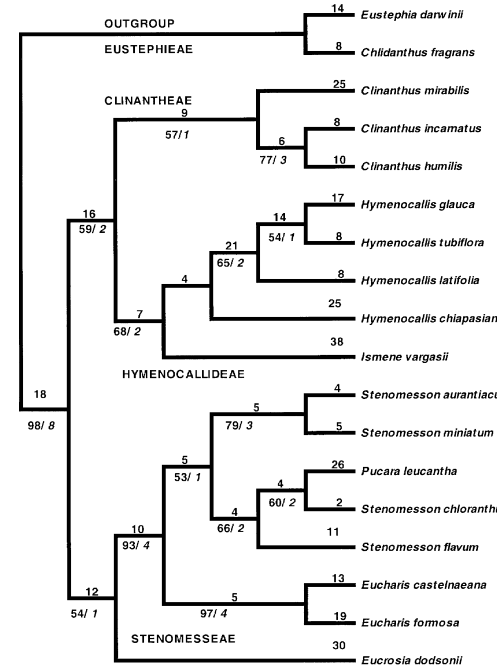

FIG. 2. One of three equally most parsimonious trees found by a cladistic analysis of the plastid *atpß-rbcL* spacer region plus a binary coded gap matrix across 18 species of the Amaryllidaceae. Numbers above branches are branch lengths. Numbers below branches are bootstrap support percentages and decay indices (italic).

son 1996). The cut-off bootstrap value is 50%. A bootstrap value greater than 75% was considered good support, 65–75% was designated moderate support, and less than 65% as weak (Meerow and Snijman 2001; Meerow et al. 2002). A branch and bound search was implemented for each constraint statement postulated by TreeRot. A minimum DI = 2 was considered to represent good support for a clade.

We also applied Bayesian analysis (Huelsenbeck et al. 2001) to each sequence matrix, in order to approximate a bootstrap of maximum likelihood estimates of the phylogenetic relationships, and check for congruence with the results of parsimony analysis. The program MrBayes v. 3.04 (Huelsenbeck and Rohnquist 2001) was used for this purpose. We first determined which model of nucleotide substitution to impose on our data with ModelTest v. 3.06 (Posada and Crandall 1998). We used the Akaike information criterion (Akaike 1974) to choose the model with the best fit. We then ran 500,000 generations of four simultaneous Markov chains with Mr. Bayes, retaining the tree from every 100th generation (i.e., a total of 5000 trees, less the trees produced before log likelihood scores stabilized) from which a 50% majority rule consensus tree was constructed. In each case, the log likelihood scores stabilized after 10,000 generations of Bayesian analysis, but we dropped the trees from 20,000 generations (200 of 5000) in constructing the 50% majority consensus trees. The results of the Bayesian analyses are reported as ''clade credibility'' (CC) scores, i.e., the percentage of trees sampled where a given clade is resolved, which is equal to the posterior probability of the clade existing (Huelsenbeck and Rohnquist 2001). Only CC scores in excess of 50% are shown in our trees (Figs. 1–5).

RESULTS

Plastid* atpß-rbcL *Spacer. The *atpß-rbcL* spacer matrix yielded 37 parsimony informative characters out of a total of 1276. The percentage of total matrix cells coded as missing was 22.5%. A branch and bound search found four equally parsimonious trees of length = 232, consistency index (CI) = 0.93 and retention index (RI) = 0.80). The topology (Fig. 1) is congruent with the major clades of Andean genera resolved in the large ITS analysis of American Amaryllidaceae by Meerow et al. (2000), *Pucara leucantha* and *Stenomesson chloranthum* are sister species with a bootstrap of 63% and decay index (DI) = 1. The nucleotide substitution model that best fit the *atpß-rbcL* spacer data was the Kimura 3-parameter model with unequal base frequencies with gamma distribution (K81uf+ G; Kimura 1981), which was approximated in Mr. Bayes with the settings nst = 2. The ''Pucara'' clade within Stenomesseae received a clade credibility score of 99% (Fig. 1). When a binary gap matrix for the spacer region was added to the sequence alignment, a total of 86 characters were parsimony informative out of 1406 total. Three equally parsimonious trees were found, of length = 420, CI = 0.82 and RI = 0.69 (Fig. 2). *Pucara leucantha* still resolves as sister species to *Stenomesson chloranthum* but with weak bootstrap support = 60

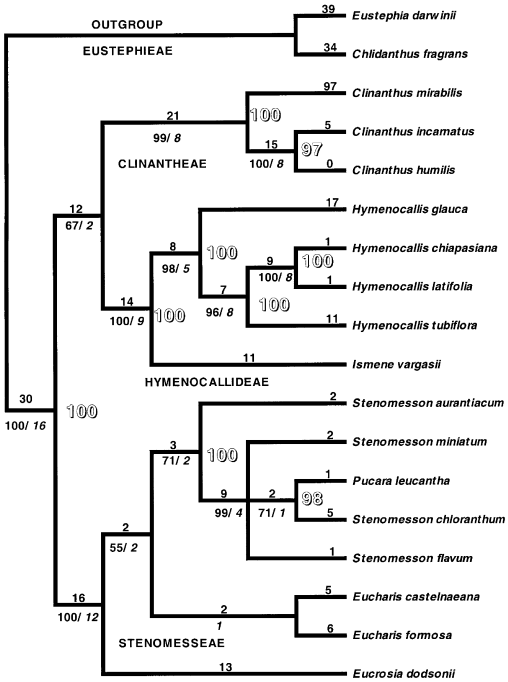

FIG. 3. Single most parsimonious tree found by a cladistic analysis of the nrDNA ITS region across 18 species of the Amaryllidaceae. Numbers above branches are branch lengths. Numbers below branches are bootstrap support percentages/decay indices (italic). Large shadowed numbers adjacent to nodes are ''clade credibility'' scores from 500,000 generations of Bayesian analysis.

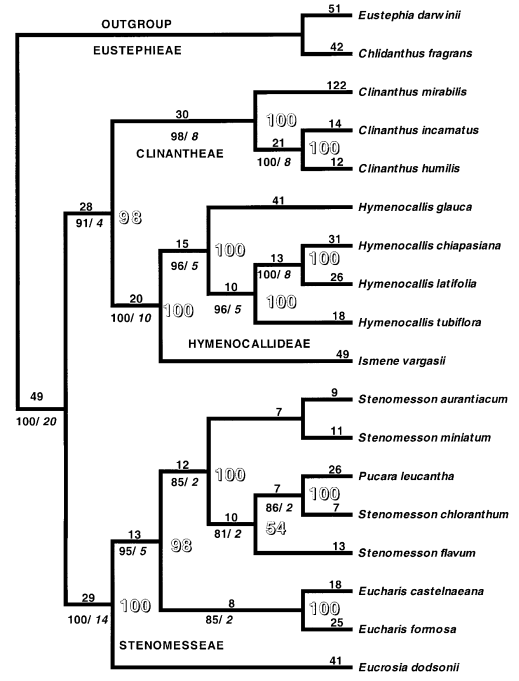

FIG. 4. Single most parsimonious tree found by a cladistic analysis of combined plastid *atpß-rbcL* spacer and nrDNA ITS across 18 species of the Amaryllidaceae. Numbers above branches are branch lengths. Numbers below branches are bootstrap support percentages/decay indices (italic). Large shadowed numbers adjacent to nodes are ''clade credibility'' scores from 500,000 generations of Bayesian analysis.

(though DI = 2). *Stenomesson flavum* resolves as sister to the *Pucara* clade with a bootstrap = 66 and DI =2. *Pucara* and its sister species are embedded within a monophyletic *Stenomesson* clade (bootstrap = 53, DI = 1).

ITS. Of 646 total characters, 129 were parsimony informative. The percentage of total matrix cells coded as missing was 6.5%. A single most parsimonious tree was found by the branch and bound search, of length = 401 steps, CI = 0.85 and RI = 0.85 (Fig. 3). *Pucara leucantha* resolves as sister species to *Stenomesson chloranthum* with bootstrap = 71 and DI = 1, nested in a clade with *S. miniatum* and *S. flavum* with bootstrap = 99 and DI = 4. *Stenomesson* is monophyletic with a bootstrap = 71 and DI =2. For the Bayesian analysis, a general time reversible model with a proportion of invariant sites (GTR + I; Lanave et al. 1984) was the model that best fit our data. Clade credibility for the sister relationship of *P. leucantha* and *S. chloranthum* was 98%, nested within a clade including *S. miniatum* and *S. flavum* (CC = 100%). *Stenomesson* was monophyletic with a CC = 98%.

atpß-rbcL *Spacer and ITS.* The partition homogeneity test suggested that the two sequence matrices were largely incongruent (P = 0.01). However, if one compares the trees supported by each of the two gene regions (Figs. 1, 3), it is clear that the incongruity is concentrated in the more terminal branches. The same main clades are resolved by both sets of data. The combined sequence matrix yielded 166 parsimony informative characters out of 1922 total. Branch and bound search found a single tree of length = 637 steps, CI = 0.87 and RI = 0.83 (Fig. 4). *Pucara leucantha* and *Stenomesson chloranthum* are again sister species, but with a bootstrap of 86 (DI = 2), within the same clade resolved by ITS alone (bootstrap = 81, DI = 2). Bootstrap support for a monophyletic *Stenomesson* rises to 85 (DI = 2). The Bayesian consensus tree of 4800 sampled trees from the analyses is highly congruent with the parsimony analysis. *Pucara leucantha* and *Stenomesson chloranthum* are sister species in a clade with *S. flavum* and *S. miniatum* with a CC= 100% in both cases. While the sister relationship of *S. flavum* to the *Pucara* subclade has a CC of only 54%, *Stenomesson* is monophyletic with a CC of 100%. Adding the *atpß-rbcL* spacer gap matrix to the combined sequence matrix raised the number of parsimony informative characters to 215, and resulted in two equally parsimonious trees of 828 steps, CI = 0.83, RI = 0.78 (not shown), differing only in the resolution of *Stenomesson aurantiacum* and *S. miniatum* (sister species in one tree; forming a grade

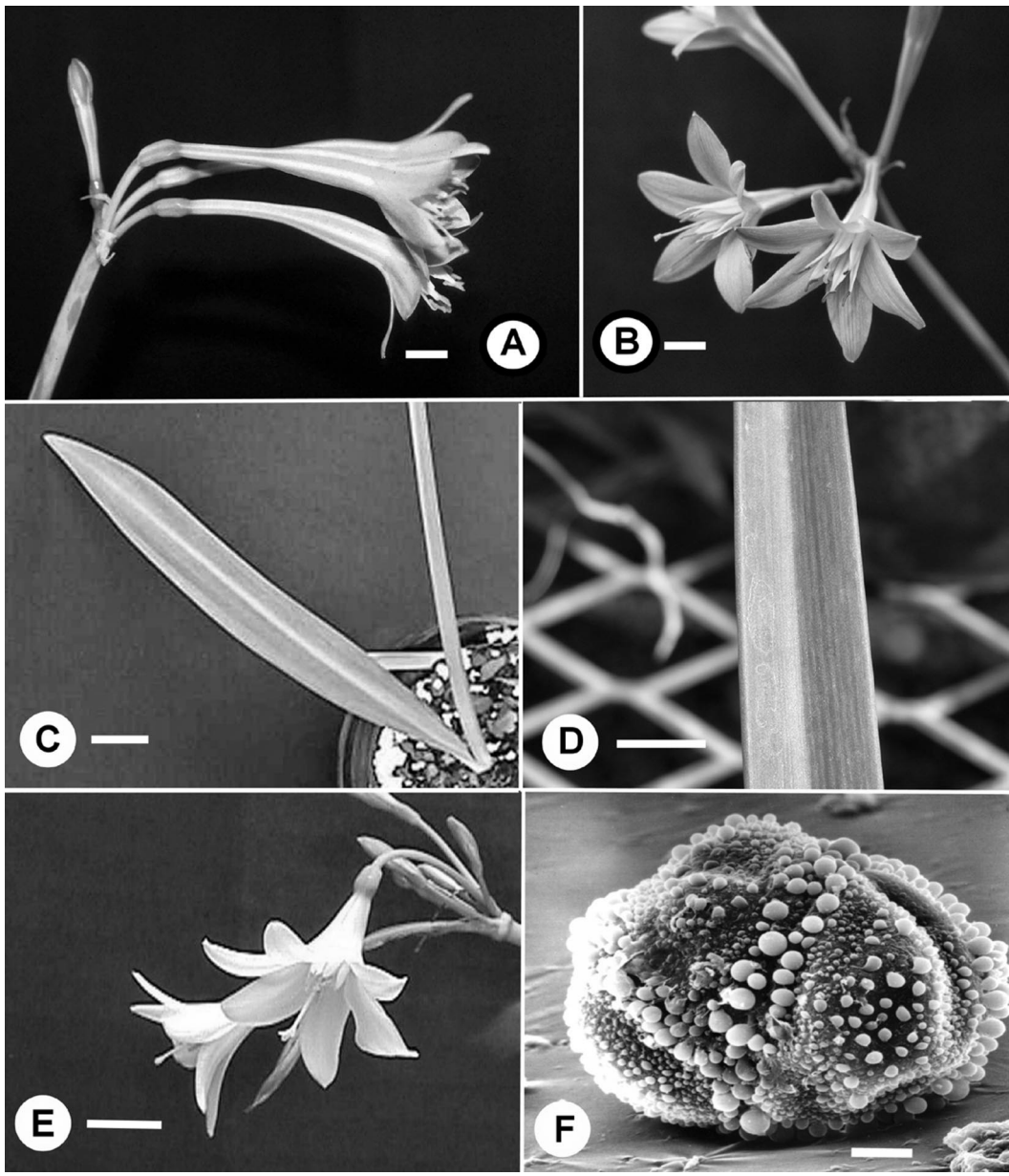

FIG. 5. *Stenomesson chloranthum* and *S. leucanthum*. A–B. Flowers of *S. chloranthum*. A. *Meerow 2520* (FTG). B. *Meerow 1155* (FLAS). C. Leaf of *S. leucanthum* (*Meerow 2522,* FTG). D. Leaf detail of *S. chloranthum* (*Meerow 2520,* FTG). E. Flowers of *S. leucanthum* (*Meerow 2522,* FTG). F. Pollen tetrad of *S. chloranthum* (Meerow 1155, FLAS). Scales A–E = 1 cm, F = 5 μm.

with the *Pucara* subclade in the other). Other than this, the tree topologies were the same as the one tree found without the gap matrix added to the sequences.

DISCUSSION

The case for treating *Pucara* as part of the larger genus *Stenomesson* is unambiguously supported by both plastid and nuclear non-coding sequences, whether analyzed by parsimony or likelihood. Both *P. leucantha* and the newly described *S. chloranthum* have shortly sub-petiolate leaves with a well-developed midrib, which is characteristic of the genus *Stenomesson*, the limits of which were re-assessed by Meerow et al. (2000) on the basis of nrDNA ITS sequences. The most significant synapomorphy of *P. leucantha* and *S. chloranthum* is the exclusive presence of pollen tetrads at anthesis (Fig. 5f), a character state that is not known to occur in any other species of the genus, or within

the entire family. Ravenna (1972) denoted the alternating position of the free staminal filaments in *P. leucantha* (those opposite the outer tepals are inserted at the rim of the staminal cup; those opposite the inner tepals, below the rim) and its tri-lobed stigma as the main basis for generic recognition. While *S. chloranthum* has a less complex androecium, polymorphism of staminal cup morphology within the Andean petiolate-leafed clade of Amaryllidaceae has been demonstrated within the limits of a single species (Meerow 1989). Transfer of *P. leucantha* into *Stenomesson* is thus warranted. *Stenomesson leucanthum* is the only white flowered species of *Stenomesson* so far known. Nothing is known about the pollination biology of the genus.

No other *Stenomesson* species from the interior of Peru have ever been found below about 2000 m elevation. Low elevation species have only before been recorded from the coastal lomas of Peru, where maximum temperatures approximate those of Andean high elevations. The same general region in Peru where *S. chloranthum* and *S. leucanthum* are found is also host to the three species of the bizarre, green-flowered, succulent-leafed endemic genus *Rauhia*, which resolved in a different sub-clade of the tribe Stenomesseae in Meerow et al.'s (2000) ITS phylogeny. The geographic concentration of such novelties suggests that the area bordered by the lower Río Utcubamba and middle Río Marañón was a hotspot for diversification in the tribe Stenomesseae as the Andes rose to their present position.

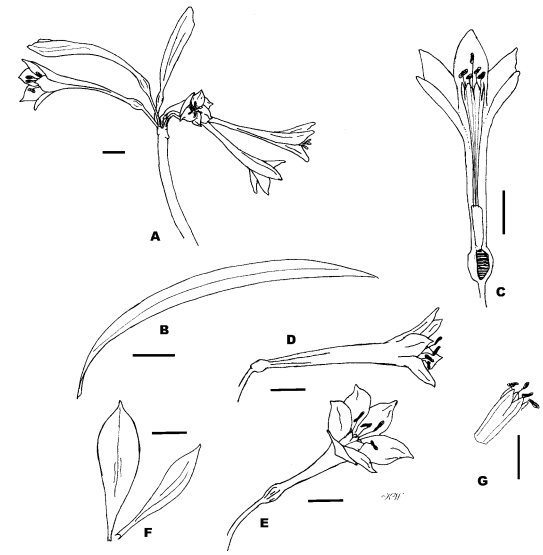

FIG. 6. *Stenomesson chloranthum* (*Meerow 2520,* FTG). A. Inflorescence. B. Leaf. C. Longitudinal cross section of flower. D. Longitudinal view of flower. E. Oblique view of flower. F. Tepals, outer (right), inner (left). G. Staminal cup. All scales = 1 cm.

TAXONOMIC TREATMENT

Stenomesson leucanthum (Ravenna) Meerow & van der Werff, comb. nov. (Figs. 5c, e). *Pucara leucantha* Ravenna, Ann. Mus. Nat. Valparaiso 5: 85–89 (1972).—Type: Peru, Cajamarca, Jaen, San Antonio (km 81—Pucara), 990 m, 12 Oct 1965, *Sagástegui 5850* (holotype: HUT).

Representative Specimens Examined. PERU. Amazonas: Pedro Ruiz to Chachapoyas, 1500 m, 24 Apr 2002 (pressed from bulbs collected by H. van der Werff s. n.), *Meerow 2523* (FTG); Utcubamba Valley between Pedro Ruiz and branch to Chachapoyas, 14 Apr 2002 (pressed from bulbs collected by H. van der Werff s. n.), *Meerow 2522* (FTG).

Stenomesson chloranthum Meerow & van der Werff, sp. nov. (Figs. 5a–b, d, f, 6).—TYPE: PERU. Amazonas: Bagua, near Pongo de Rentema on the Río Marañon, 1 km east of Olmos on the Mesones-Muro Highway, 370 m, 26 Jan 1964 (pressed from living material 23 June 1965), *Hutchinson and Wright 3782* (holotype: UC!).

S. leucantho affine sed foliis olivaceis, floribus pallide viridibus, staminibus insertis pariter margine coronae, et praesentia altitudine inferiore differt.

Representative Specimens Examined. PERU. Amazonas: Bagua Grande, 500 m, 25 Mar 2002 (pressed from bulbs collected by H. van der Werff s. n.), *Meerow 2520* (FTG). Cajamarca: Jaen, 2 km north of Chamaya on rd. to Jaen, 450 m, 7 Feb 1964 (pressed 8 Jul 1965 from living material), *Hutchinson & Wright 4123* (UC); Cajamarca: a few km outside Jaen towards San Ignacio, 600 m 8 Apr 2002 (pressed from bulbs collected by H. van der Werff s. n.), *Meerow 2521* (FTG).

Bulbous perennial herb; bulb globose, with a short (2–3 cm) neck, 2.5–4 cm diam, producing offset bulbils. Leaves 2–3, hysteranthous or emerging with the flowers, sub-petiolate, lanceolate, obtuse, 25–29 cm long, 2–2.5 cm wide, olive-green (Royal Horticultural Society Color Chart Green 137A), somewhat glaucous, with an obscure midrib on the adaxial surface (pronounced on the abaxial). Inflorescence scapose; scape terete, solid, 30–40 cm long, 7–8 mm diam proximally, 5 mm diam distally, terminated by a pseudoumbel of 5–10 flowers enclosed by two obvolute, greenish-white, marcescent bracts before anthesis; bracts ovate-lanceolate, 2.5–3 cm long, 10–11.5 mm wide at the base, acute. Perianth campanulate-tubular, pale green, 5.8–6.7 cm long (from base of tube to apex of limb); tube 3.5–4 cm long, cylindrical and 2.5–3.1 mm wide at base, abruptly dilating in the distal half to 8.3–9 mm at the throat, longitudinally striped white, limb spreading 18.5–19 mm wide. Tepals 6, in two series, outer 17.7–18.5 mm long, 8–8.5 mm wide, elliptic, acute and minutely apic-

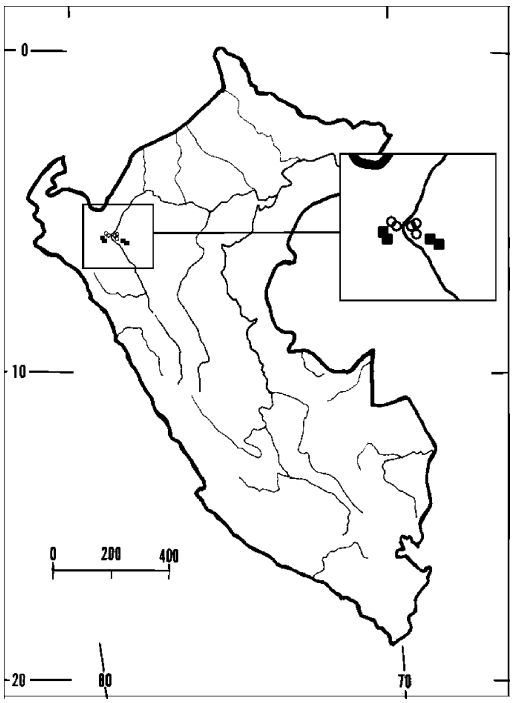

FIG. 7. Map of Peru showing distributions of *Stenomesson chloranthum* (open circles) and *S. leucanthum* (black squares).

ulate; inner 16.8–18.3 mm long, 8.3–8.9 mm wide, elliptic, obtuse, margins of both series hyaline. Stamens 6, white to greenish white, fused for the proximal 4–5 mm into a short staminal cup; free filaments broadly subulate, edentate, 8.3–8.7 mm long, 2.7–3 mm wide, abruptly tapering to 1 mm in their distal 2 mm, barely exserted from the limb; anthers ca. 3 mm long, oblong, dorsifixed, introrse; pollen white, released in tetrads (Fig. 5f). Style filiform, ca. 6 cm long, exceeding the stamens by about 5 mm; stigma trilobed. Ovary ellipsoid, 7.5–8 mm long, 4.3–5 mm wide, locules 3, ovules axile, numerous per locule, flattened and superposed. Fruit a turbinate, loculicidal capsule, turning papery at dehiscence; seeds numerous per locule, black, flat, obliquely winged. $2n = 46$.

Stenomesson chloranthum is endemic to the lower slopes of the seasonally dry interandean valleys of the Marañón and Utcubamba drainages in northern Cajamarca and west-central Amazonas departments of Peru (Fig. 7), between 350–600 m elevation, in dry scrub, often growing with *Opuntia* and other Cactaceae.

Stenomesson chloranthum can be distinguished from *S. leucanthum* by its more glaucous, olive-green leaves with a more obscure adaxial midrib (Fig. 5d), its fewer but larger green and white flowers, the more simplified structure of its androecium, and its lower altitudinal limits. The white-flowered *S. leucanthum* is not known from elevations lower than 900 m and occurs up to 1650 m elevation. The adaxial mibrib is conspicuously visible on the less glaucous leaves of this species (Fig. 5c).

ACKNOWLEDGMENTS. Karen Williams prepared the drawings of *S. chloranthum*. This work was partially supported by National Science Foundation Grants DEB-968787 and 0129179 to AWM.

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APPENDIX 1

Species, vouchers, and Genbank accession numbers (or literature citations for previously published sequences) of DNA sequences used in this paper. All vouchers deposited at FTG unless otherwise stated.

Clinanthus humilis (Herb.) Meerow—*Meerow 2442*: *atpß-rbcL* spacer AY460393, ITS Meerow et al. (2000). ***C. incarnatus*** (H.B.K.) Meerow—*Meerow 1120*: *atpß-rbcL* spacer AY460392, ITS Meerow et al. (2000). ***C. mirabilis*** (Rav.) Meerow—*S. Leiva et al. 2000* (HUT): *atpß-rbcL* spacer AY460391, ITS Meerow et al. (2000)

Chlidanthus fragrans Herb.—*Meerow 2312*: *atpß-rbcL* spacer AY460390, ITS Meerow et al. (2000)

Eucharis castelnaeana (Baill.) MacBride—*Schunke 14156* (FLAS): *atpß-rbcL* spacer AY460405, ITS Meerow et al. (2000). ***E. formosa*** Meerow—*Whitten et al. 95020* (FLAS): *atpß-rbcL* spacer AY460406, ITS Meerow et al. (2000)

Eucrosia dodsonii Meerow & Dehgan—*Meerow 1115*: *atpß-rbcL* spacer AY460404, ITS Meerow et al. (2000)

Eustephia darwinii Vargas—*Meerow 2436*: *atpß-rbcL* spacer AY460389, ITS Meerow et al. (2000)

Hymenocallis chiapasiana T. M. Howard—*T. M. Howard 1185* (MO): *atpß-rbcL* spacer AY460396, ITS AY461739. ***H. glauca*** M. Roem.—*Meerow 2433*: *atpß-rbcL* spacer AY460395, ITS Meerow et al. (2000). ***H. latifolia*** (Mill.) M. Roem.—*Meerow 2438*: *atpß-rbcL* spacer AY460398, ITS Meerow et al. (2000). ***H. tubiflora*** Salisb.—*Meerow 2440*: *atpß-rbcL* spacer AY460399, ITS Meerow et al. (2000)

Ismene vargasii (Velarde) Gereau & Meerow—*Meerow 2308*: *atpß-rbcL* spacer AY460397, ITS Meerow et al. (2000)

Stenomesson aurantiacum Herb.—*Meerow 1061* (FLAS): *atpß-rbcL* spacer AY460394, ITS Meerow et al. (2000). ***S. chloranthum*** Meerow & van der Werff—*Meerow 2520*: *atpß-rbcL* spacer AY460403, ITS AY461738. ***S. flavum*** (R. & P.) Herb.—*Meerow 2430*: *atpß-rbcL* spacer AY460402, ITS Meerow et al. (2000). ***S. leucanthum*** (Rav.) Meerow & van der Werff (*Pucara leucantha* Rav.)—*Meerow 2522*: *atpß-rbcL* spacer AY460401, ITS AY461737. ***S. miniatum*** (Herb.) Ravenna—*Meerow 1118*: *atpß-rbcL* spacer AY460400, ITS AY461736.